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14. ABSTRACT

The purpose of this project is to enable more patients to access VCA transplantation. The work in Louisville will focus on using non-invasive imaging techniques that can be used to monitor vessels and nerves in VCA recipients (Aim 6). The goal is to identify changes while there is still time to intervene. Studies are initiated using infrared imaging of ICG dye to study blood perfusion and lymphatic drainage in our hand transplant patients. We are extending our studies of vessel wall thickness using very high resolution ultrasound, and including non-invasive studies of nerve anatomy. In Aim 7 we established a rodent model to study VCA vasculopathy, both with respect to imaging modalities and what factors initiate or exacerbate graft vasculopathy. Initial IR-ICG imaging studies of graft perfusion correlate well with acute graft rejection in our animals. Finally in Aim 8 we will develop standardization of protocols and clinical monitoring and treatment for VCA targeting vascular health.

15. SUBJECT TERMS VCA, vasculopathy, animal model, high resolution ultrasound, Fluorescence angiography, immune monitoring, graft rejection, histology, standardization, hand transplant, face transplant

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INTRODUCTION: The principal objective of this project is to hone vascularized composite allotransplantation (VCA) into a useful therapeutic option for patients in need of advanced tissue reconstruction and replacement. This is a multi-institutional and multi-disciplinary project between the Louisville VCA Program, which is composed of four different institutions in Louisville, the University of Pennsylvania, the University of Maryland, and at this point, Duke University. The work in Louisville is focused on aims 6,7 and 8 of the Statement of work. Specifically in Aim 6 we will evaluate less invasive bio-imaging modalities with standard of care biopsy and peripheral blood analysis to assess vasculopathies associated with VCA in patients. In Aim 7 we will establish disease mechanisms associated with vasculopathy in pre-clinical models of VCA. Finally in Aim 8 we will develop standardization of protocols and clinical monitoring and treatment for VCA targeting vascular health. We propose to serve as the central site for the standardization of bioimaging assessment of vasculopathies in hand and face allotransplants. The ultimate goal is to expand the available options for individuals with combat-related injuries in need of complex tissue reconstruction by elevating VCA to the level of an established therapy for use in appropriately selected personnel with severe traumatic tissue loss.

KEYWORDS: VCA, vasculopathy, animal model, high resolution ultrasound, Fluorescence angiography, immune monitoring, graft rejection, histology, standardization, hand transplant, face transplant

ACCOMPLISHMENTS:

The major goals of this project are focused on Aim 6, 7 and 8 of the Statement of work, which are as follows:

Aim 6. To evaluate less invasive bio-imaging modalities with standard of care biopsy and peripheral blood analysis to assess vasculopathies associated with VCA in patients.

Hypothesis: Vascular events associated with VCA can be identified and the therapeutic intervention efficacy can be assessed using minimally invasive, bioactive contrast imaging of the transplant vasculature. To test the hypothesis, we will perform vascular assessments in existing and new VCA patients in the Consortium using our bioimaging modalities. Image findings will be correlated with clinical course, rejection activity, biopsy results (including genomic analysis), and blood-based measurements including flow cytometric and cytokine analyses, with specific emphasis on humoral immune responses.

Performing Institution: Louisville

Task 1. To establish and maintain a database of imaging data and clinical follow-up including immune monitoring assays.

Task 2. To perform imaging clinical and immune monitoring of subjects as well as historical controls for comparison of rejection episodes, humoral immune status and mature lineage and cytokine analysis of peripheral blood Regulatory review and approval process (months 1-4).

Subtask 2.1. To collect data regarding non-histologic indices of rejection such as hand volume (edema), presence of rash or erythema, and level of involvement, i.e. localized or generalized involving a named (i.e. 25%) percentage of the allograft dorsal or ventral surface.

Subtask 2.1. To perform immune monitoring assays on peripheral blood lymphocytes at rejection and following resolution.

Aim 7. To establish disease mechanisms associated with vasculopathy in pre-clinical models of VCA. Hypothesis: VCA-associated macro- and microvasculopathies are due to chronic and multiple acute rejection activities, and can be exacerbated to confluent aggressive vasculopathy by non-alloimmune triggers. We will perform vascular imaging assessments utilizing mouse preclinical models of VCA in experiments with defined rejection regimens. In addition, we will use vascularized composite autograft models to evaluate the effects of inflammation and antirejection medications in the absence of active rejection. Our evaluations will include molecular

and histologic analyses in combination with imaging-based measurements, including those utilizing already developed and tested targeted bioactive contrast agents for use in assessing vascular status.

Performing Institution: Louisville (in collaboration with Penn, Emory and Maryland)

Task 1: To perform osteomyocutaneous (hindlimb) allogeneic VCA in rodents (Regulatory review and approval process.

1. ***Task 2:*** To establish baseline and experimental imaging standards in rodent model
2. **Subtask 2a:** To transplant groups of animals and follow and image over a 28-90 day period
3. **Subtask 2b:** To perform immunologic and histologic analysis of VCA recipients
4. ***Task 3:*** To perform murine transplants involving pharmacological and genetic perturbations to allo-rejection
5. **Subtask 3.1:** To transplant groups of animals evaluating experimental interventions with imaging
6.
7. **Aim 8– To develop standardization of protocols and clinical monitoring and treatment for VCA targeting vascular health**. We propose to serve as the central site for the standardization of (a) bioimaging assessment of vasculopathies in hand and face allotransplants, and (b) Collaborate with consortium members in submission of biorepository samples and digitization of existing Hematoxilin and Eosin slides of clinical VCA biopsies.
8. **Focus areas:** Clinical Monitoring of Composite Tissue allotransplant recipients, and Established Practice and Protocol
9. **Performing Institution:** All sites
10. ***Task 1*****:** To establish reproducible and HIPAA compliant protocols for UBM, MLDI and SPY imaging in VCA recipients (Regulatory review and approval process (1-4). Data collection

11. ***Task 2:*** To implement and maintain database and data sharing protocols for clinical and experimental animal data with consortium members
12. ***Task 3:*** To participate with consortium members in submission of historical and prospective VCA patient histological slides and biopsy samples for digitization using the Aperio system.

What was accomplished under these goals?

Briefly, in the second year of this grant, we have established our rat osteomyocutaneous flap model, as well as procedures for the ultrasound imaging analysis of the vessels in the animal model and immunomonitoring including flow cytometry and functional testing.

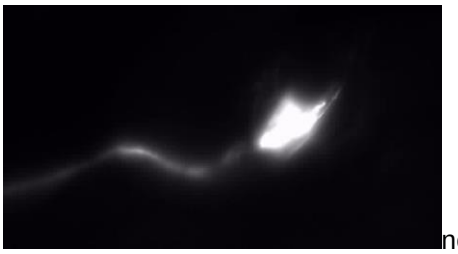
In the second year of the grant we submitted an amendment to our current hand transplant protocol which requests permission from current and prospective hand transplant recipients to send specimens and information to the VCAci consortium, which is located at Duke University. This amendment has been reviewed, and minor modifications to the consent form were requested by our IRB. These changes have been made and submitted for review to the IRB.

In the second year of the grant we were able to purchase new software that will reduce the subjectivity of measuring artery and vein wall thickness by biomicroscopy ultrasound, especially the arterial wall intima in our hand transplant patients. This software has been installed and study personnel are in the process of re-analyzing the backlog of data acquired from previously transplanted patients.

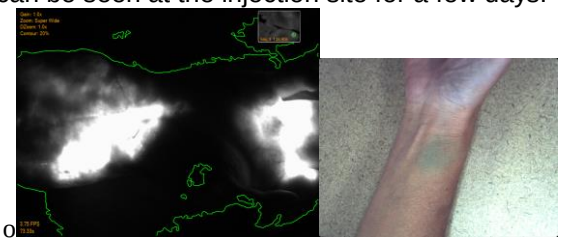
In addition to the ongoing monitoring of all of the current hand transplant patients, as well as collecting images on subject who are being screened for transplant, we have initiated studies that will use the Vevo 2100 to monitor the medial and ulnar nerves. Using the very high resolution probes we are able to image the nerve bundles within the

nerve. With the new software upgrade, we will be better able to quantitate the number and size of the bundles. As the study progresses we hope to be able to correlate changes in function with changes in the images collected.

Figure 1: pilot study of ICG subcutaneous injection and imaging



in a normal control (top) and a hand transplant recipient (lower). Note the linear drainage pattern in the normal control vs. the patchy drainage patter in the transplant (lower image) Using 100 ul of ICG, a greenish stain can be seen at the injection site for a few days.



In addition to the use of the use of the LUNA unit to study vascularity of the transplanted hand, we will also use this technology to image lymphatic drainage of the hand. We have initiated a study of sub-cutaneous injection of small quantities (less than 100 ul) of the dye and then imaging the injected site over hours and days to follow the clearance of the dye by the lymphatic system in normal controls. We realized that most of the parameters for lymphatic drainage reported in the literature were obtained from subjects with impaired lymphatic drainage such as breast cancer patients. In the second year of this study we designed a sub-study to inject small amounts of indocyanine green sub-cutaneously and monitor drainage of the dye through the lymphatics present in the upper extremity of normal controls. We performed a pilot study and have prepared a protocol for a larger study of 50 controls and 50 hand surgery patients which will include our hand transplant recipients. This study is currently under IRB review. In a pilot study we found that ICG dye was cleared in a linear fashion in the one normal control studied, and in a patchy fashion in one of our hand transplant recipients. This study will be important to establish normal parameters for the assessment of transplanted subjects.

Although outside the funding of the current grant, an important part of our study is our success in enrolling and transplanting VCA recipients. We are putting significant effort into finding and screening potential candidates. To date, the majority of candidates who approach our program have failed our screening procedures. However, those programs with active clinical VCA programs understand how critical the screening process is to achieving good outcomes. Strict adherence to our inclusion and exclusion criteria are paramount to the success of this project as well.
The current working summary of potential and pending candidates for hand transplantation is as follows:
Male age 31, excellent candidate, approved for listing, waiting on workman's comp settlement. Workman's comp has denied twice in the last quarter. Patient is now seeking legal assistance to obtain Workman's compensation coverage for immunosuppression.

Male age 32, excellent candidate, family situation changed, subject still interested in transplant once family issues are more manageable.

Female age 37: Was listed for bilateral hand transplant, but subject's PRA increased from 79% to over 90%. Subject made decision to withdraw from the study after waiting for over three years for a suitable donor.

Female age 68: Currently listed for bilateral hand transplant, 0% PRA

Female age 39: Cleared medically, financially and socially for transplant, but will not be listed until BMI is reduced from 43 to 32.

In addition, in year 2 of the grant (specifically in November of 2014) we transplanted the ninth recipient to receive a hand transplant in Louisville. The team performed a unilateral hand transplant. Unfortunately, the graft failed for technical reasons on day 5

post transplant. This serious adverse event was reported to U of L IRB, HRPO (which has oversight through a grant with AFIRM II) , our DSMB.

To summarize the case, on 11/25/14 during the transplant there was oozing during the procedure and the patient required 13 units of PRBC intraoperatively. The veins of the recipient were smaller than anticipated and in some cases significantly smaller than the donor veins. The surgeons attempted to connect as many veins as possible and an additional vein graft was placed to improve outflow. The hand color and outflow was initially good, but the proximal end of the graft became swollen and developed congestion, possibly as a result of ischemic reflow phenomenon. Venous pressure continued to be high and oozing continued. The patient required an additional 4 units of PRBC over 11/28 and 11/29. On 11/30/14, the patient presented with severe swelling, dark color and necrosis of skin in proximal end of graft. The patient was taken back to the OR and exploration revealed necrosis of muscle and tendons, especially proximally, and a thrombotic vein within the graft. After consulting with the family, a decision was made to amputate the allograft. The patient tolerated the amputation well and was released from the hospital on 12/5/14.

The surgical team met and reviewed the timeline of when the patient received a block and was sent to the OR for preparation, when the graft was amputated, the recovery, and the discharge of the patient. In the course of dissection of the recipient arm in preparation for attaching the transplant, the team realized that the internal damage to the patient's stump was greater than anticipated. As a result, a better surgical approach for this patient might have been an above the elbow rather than the very proximal below the elbow approach that was taken. In future procedures with amputations at this proximal level, the team will plan for both possibilities. Additionally when transplanting at this level, the team will plan on disarticulating the donor limb near or at the shoulder to allow for procurement of as much tissue as is needed to reconstruct the recipient.

The peri and post operative management of the patient was also reviewed. The warm ischemia time was reviewed. The time from the tourniquet placement at the donor hospital (2:12 AM) to starting the dissection of the donor hand in Louisville (6:30 AM) was shorter than most of our previous hand transplants. The time of the surgery was also the second fastest at about 10.5 hours to closing of the skin. The blood loss and requirement for 13 units of PRBC was higher than in most of the previous transplant and was attributed to the intraoperative oozing. Early in the morning of 11/28, when it was clear the graft was in trouble, a skin biopsy was taken to rule out graft rejection. This biopsy came back a Banff Grade I (attachment 1) suggesting aggressive cellular rejection was not the problem.

Histology of the amputated graft showed significant necrosis, but also arteritis with fibrinoid necrosis in several of the arteries. This could be consistent with humoral rejection (attachment 2). However, the crossmatch done on serum from the day of transplant was negative (attachment 3). Also, the patient was immunosuppressed and received three doses of ATG, which reduced circulating CD2 and CD3 T cells by >95%. Additionally we tested recipient serum for DSA on 12/4/14, and this sample was also negative, except for one antigen at a low MFI of 1700 (attachment 4). As the patient had received 25 units of PRBC between 11/25 and 11/30, the HLA lab felt even that antigen was not relevant.

In conclusion, our review suggested that the graft loss was due to ischemia and resulting necrosis of the transplanted hand in the proximal end of the transplant. This ischemia was likely due to swelling and resulting venous thrombosis as a result of inadequate venous outflow in the proximal end of the transplant. Surprisingly the fingers and the distal portion of the hand appeared to be fairly well perfused, and this contributed to an underestimation of the poor venous drainage in the proximal end of the graft. The major change to be implemented in future transplants is additional planning for unexpected technical challenges and damage in the recipient stump.

Additionally the team will procure sufficient donor length to allow modification of the reconstruction plan.

The patient returned to Louisville on two separate occasions for follow up after discharge from the hospital. The patient is seeking re-transplantation. At this time we are aggressively pursuing alternative therapies, including targeted muscle innervation. If all other avenues are exhausted, and our team is convinced the patient really does want a second hand transplant, he may be considered. At this time we do not have evidence that the patient was sensitized by the transplant, but we will continue to monitor him for evidence of donor specific antibody production.

The IRBs, DSMB, and the medical monitor accepted the report and did not suggest changes to the study.

Analysis of previously collected Vevo 2100 data

In year 2 of the project we organized the previously collected Vevo 2100 data and have begun re-analyzing the data using the new VevoVasc software. We have over 200 timepoints from hand transplant recipients, candidates and normal controls to reanalyze, most with measurements of the radial, ulnar, palmar arch and thumb and ring finger digital arteries. We will analyze with respect to intimal medial layer thickness, and intermittently compare patient and control data to determine the usefulness of lumen diameter, and vessel wall elasticity and stiffness. This is a very interesting but challenging analysis as there is little previous data in humans to compare our results to.

Implementation of Aperio digitalization of biopsy histology

In year 2 of the proposal we successfully established accounts at the Brown Cancer Center to access the Aperio Digital Slide Scanner. The system allows us to log on to the system and access images for sharing with our collaborators at Duke, U Penn and U Maryland. Dr. Chilton has scanned the first set of slides. Using a Leica Aperio

Imaging microscope, we are now in in the process of digitizing the biopsy slides from our hand transplant patients. Each slide received from the pathology lab has 2-5 serial tissue slices stained for cellular infiltrate analysis using hematoxylin and eosin dyes (H&E). Some of these tissue sections are in better physical shape than others, therefore, the best three (if available) sections per slide are acquired as individual digital images at 40X magnification. The pathology department's slide label is used as the file

Figure 2

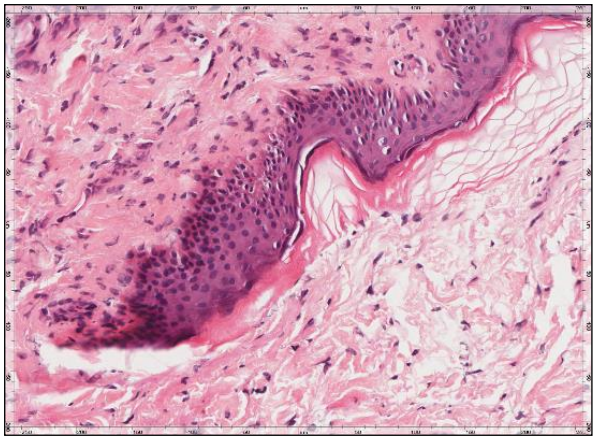
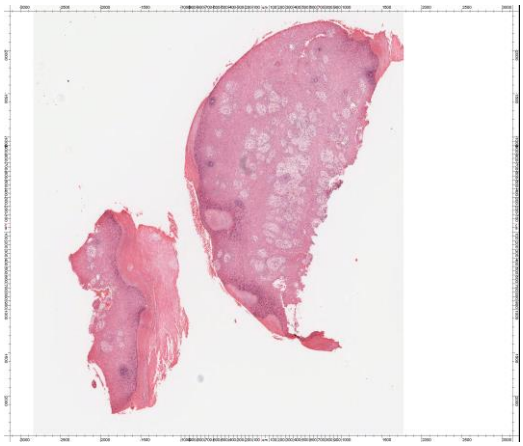


Figure 3



name followed by "#n" where the n=the individual section being scanned from that slide. The system also compiles the following data: patient initials, date of sampling, specific tissue and location sampled (i.e left dorsal hand, skin or muscle), type of staining (thus far, only H&E), and clinical score (Baniff rejection score). This information is compiled into a database and is accessed via Spectrum software on a secure server. With these images digitized and available to us, we then retrieve them and analyze and save as TIF files for sharing with the consortium. Figure 2 is an example of an archived skin biopsy acquired this past month. The 40X magnification has been increased to 80X using the Imagescope software for anlaysis. There are approximately 400 clinical transplant H&E slides as well as selected slides of the rat hind-limb project that are designated for scanning and sharing with the consortium members.

Hand transplant recipient specimen archive and immunomonitoring

In year 2 of the protocol we have implemented new immunomonitoring procedures which include extra flow cytometric analysis of circulating peripheral blood populations and archiving of extra cells, serum, plasma and RNA Paxgene samples.

8-color Peripheral blood phenotyping panel—

In year 2 of the grant we focused on putting together an 8-color immune cell phenotyping panel in order to be able to follow patient samples over time and in relation to clinical course. The following panel allows us to follow the percentage of granulocytes, monocytes and lymphocytes contained within the peripheral blood.

Table 1	**optimal dilution**
CD14 FITC	40
CCR4 PE	300
CD4 PerCP-Cy5	588
CD25 PE-Cy7	144
CD56 APC	36
CD8 APC H7	588
CD3 V450	288
CD19 V500	288

This combination of markers allow us to differentiate at least nine cell populations (Table 2,3) employing negative gating as well as positive. This strategy cleans up the analyses and allows for more consistent results (Table 3), which we hope will allow us to detect differences between samples in which could be indicative of the status of the graft. From the initial samples we have compiled the phenotyping data we have been able to acquire on the patients as they come in for annual evaluations as well as for the current individual who has been listed and waiting for a suitable donor.

Table 2	positive gating	negative gating
T cells	CD3+	CD19neg
CD4+ T cells	CD3+ CD4+	CD19neg
Tregs	CD3+ CD4+ CD25+ CCR4+	CD19neg
CD8+ T cells	CD3+ CD8+	CD19neg
CD8 "Tregs"	CD3+ CD8+ CD25+ CCR4+	CD19neg
NKT cells	CD3+ CD56+	CD19neg CD14neg
Monocytes	CD14+	CD3neg CD19neg CD56neg
NK cells	CD56+	CD3neg CD19neg CD14neg
B cells	CD19+	CD3neg

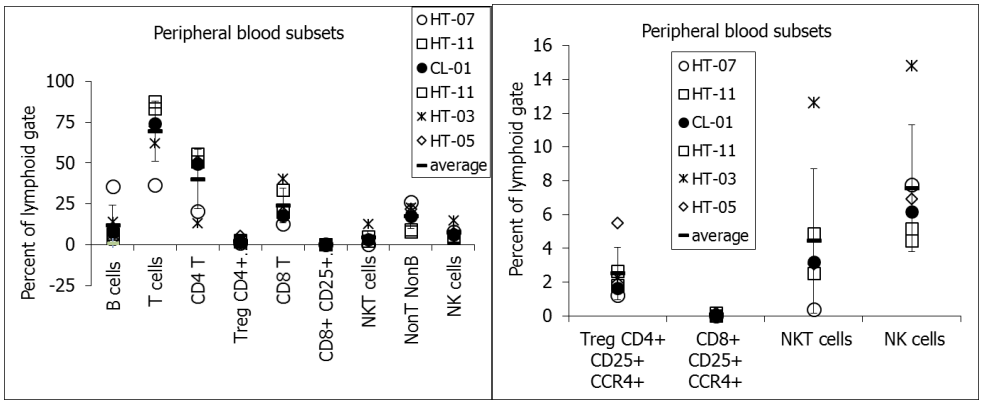


Figure 4. Results from 8-color peripheral blood phenotyping panel are shown for individual hand transplant patients (HT) and control (CL) individuals. The average ± standard deviation is indicated.

Thus far, we have two observations which will be followed. The

first is an increase in NK T cells and NK cells in the lymphocyte gate of patient 3, who is currently dealing with severe chronic rejection, manifested in the skin and adnexal tissues. As shown in Figure 4, right panel, a single sample has 3-fold and 2-fold more NK T cells and NK cells, respectively, than mean of the grouping, and easily clears the standard deviation. The second observation is an interesting increase in the percentage of regulatory T cells (Treg) in the periphery of a single patient (Fig 5, right). The Treg percentage is approximately double in this patient compared to all others tested. The mean ± standard deviation is quite similar for this population in the blood with the single control sample (closed circle, CL-01) hitting the center. We will continue to monitor patients with this panel as well as the next.

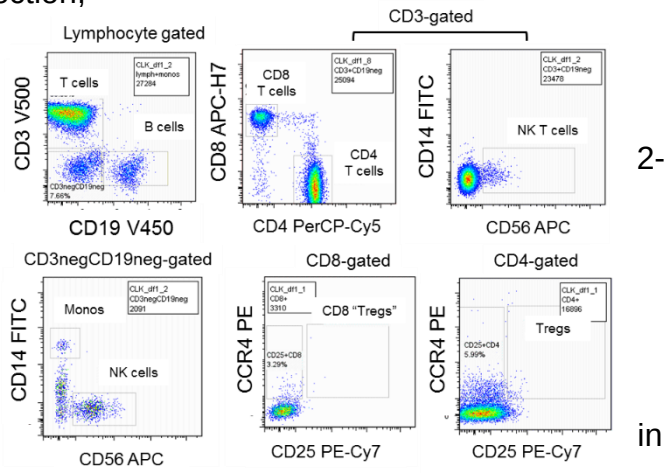


Figure 5: T regulatory subsets

Table XX. BD/Pharmingen Human Naïve/Memory T Cell Panel	dilution factor
CD45RA FITC	200
CD8 PE	50
CD4 PerCP-Cy5	200
CD197 (CCR7) AF647	200
CD3 APC H7	200

5-color T cell Memory vs. naïve phenotyping panel—In addition to the lymphocyte phenotyping panel, we have also started to use the naïve vs. memory T cell panel as sold by BD/Pharmingen (with the addition of CD8 PE), also hoping to follow trends in T cell phenotypes in patients. As shown in Figure 6, the variation within these populations is greater than with the phenotyping panel above. However, this may be due to groups of data. If this is the case, then acquisition of more individual samples will help to elucidate any potential findings. As with the 8-color phenotyping, we will continue to monitor the blood for indication of immune status within our patients using these stains.

Archiving of patient samples— Regrettably, the -140 freezer used to store long term samples malfunctioned in 2013 and the majority of the archived samples from the current hand transplant recipients were lost. In Year 2, we have taken additional steps to install monitors on the freezers and move the archive storage to freezers on emergency back up power. As patients arrive for their annual evaluations, or if samples are drawn for other purposes, we have been collecting serum, plasma and peripheral blood mononuclear cells. We will continue to archive patient samples with the plans to monitor development of donor-reactivity. In addition, we have more recently begun to freeze down peripheral blood in RNA PAXGene tubes (BD/Qiagen). These tubes contain a reagent that stabilizes intracellular RNA and protects it from degradation when frozen.

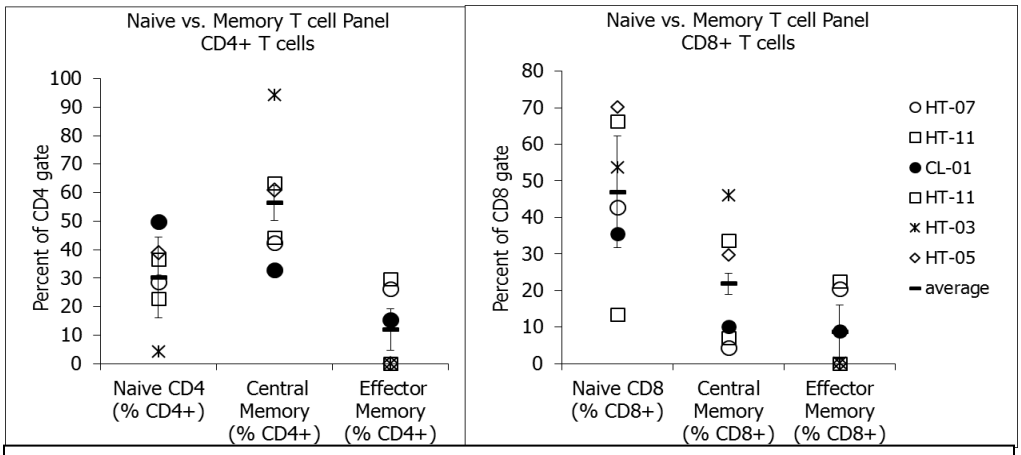


Figure 6. Percentage of naïve and memory T cell populations within the CD4 (left panel) and CD8 (right panel) T cell population. Data for individual hand transplant patients (HT) and control (CL) individuals. The average ± standard deviation are also shown by black line with error bars.

CFSE-based MLR for assessing allogeneic responses in patients—In order to monitor for T cell-specific allogenic responses in our patient population, we want to establish a CFSE-based mixed lymphocyte reaction (MLR) assay. To this end, PBMC from a control individual were isolated and labeled with CFSE following standard protocols. These cells were then incubated in triplicate at 37C in culture medium alone or in the presence of either syngeneic or allogeneic Mitomycin C-treated stimulator cells. The

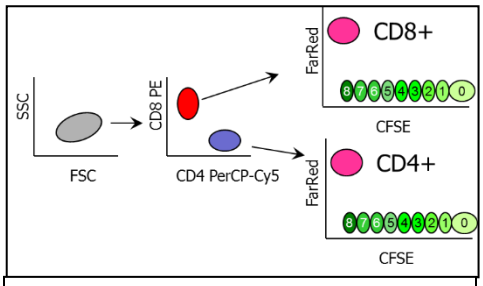


Figure 7. Schematic diagram of FACS analysis for Human CFSE-based MLR

stimulator cells were also labeled with FarRed cell tracker dye that was used in the Flow cytometric analysis to eliminate those cells in the proliferation profile. After 5 days in culture, cultures were harvested and stained for CD4-PerCP and CD8-PE. Cells incubated in medium alone were used to set the control marker, since any proliferation that occurred in that population was carry-over from in vivo activation. Although only 1.7% of the responders cultured with syngeneic (or auto) stimulators proliferated in culture, 6.15 of the responders proliferated in response to allogeneic stimulator cells. Given the low frequency of specific T cell clones in non-sensitized individuals, this is indeed a significant increase in proliferation, showing that the assay is ready to use when a sample comes available. In addition, the flexibility of the flow cytometric analysis will allow us to incorporate functional analysis in these cultures, such as IFN□ or IL-17 intracellular staining.

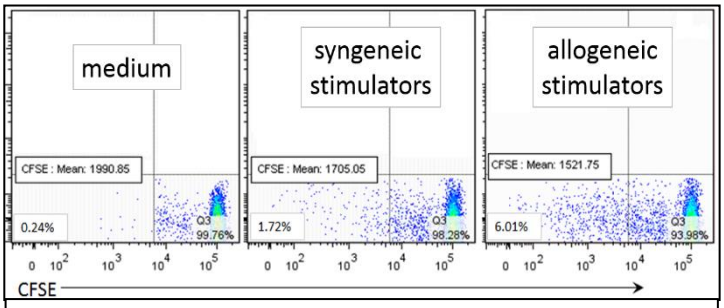


Figure 8. Representative proliferation data from CFSE-labeled PBMC cultured in medium alone or with syngeneic or allogeneic stimulator cells. As CFSE-labeled cells divide, they halve the fluorescence of CFSE between daughter cells. Therefore, further decrease in intensity reflects successive divisions.

In addition to studies focused on improving and standardizing monitoring of VCA recipients in Aim 6 and 8, Aim 7 will address the hypothesis that VCA-associated macro- and microvasculopathies are due to chronic and multiple acute rejection activities, and can be exacerbated to confluent aggressive vasculopathy by non-alloimmune triggers. We will perform vascular imaging assessments utilizing rodent preclinical models of VCA in experiments with defined rejection regimens. In addition, we are using vascularized composite autograft models to evaluate the effects of inflammation and antirejection medications in the absence of active rejection. The work in this Aim is guided by 3 tasks, the first two of which are relevant to this year 2 progress report.

Task 1: To perform osteomyocutaneous (hindlimb) allogeneic VCA in a rodent model

Task 2: To establish baseline and experimental imaging standards in rat model

Task 3: To perform rat transplants involving perturbations to allo-rejection

Task 1: To perform osteomyocutaneous (hindlimb) allogeneic VCA in rat.

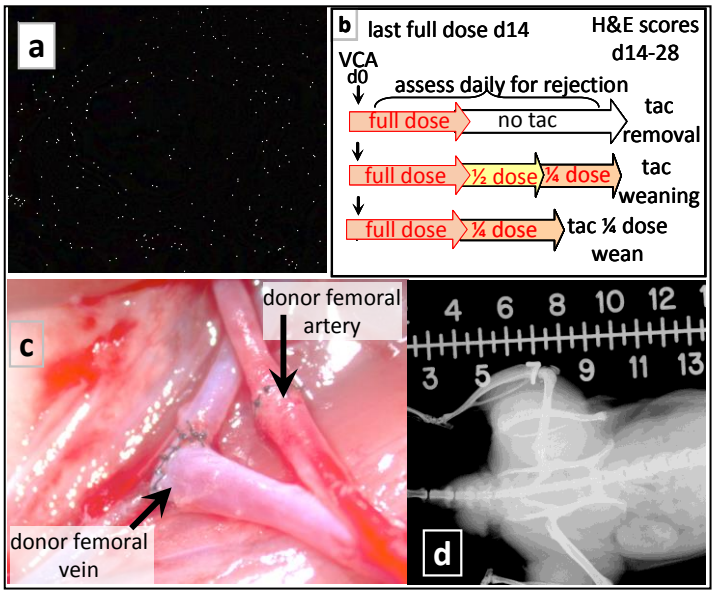


Figure 9

In the second year of the grant we have made significant progress on our rat model of VCA. In preparation of the graft, a vascular leash containing both the femoral artery/vein pair and distal iliac artery/vein regions was isolated. Furthermore, the musculature of the lower hindlimb was resected to leave only the gastrocnemius remaining, the primary muscle group supported by the isolated vascular leash. Care was taken in preparing the graft to preserve artery perforators supporting skin perfusion. The graft is transplanted in the inguinal pocket of the recipient with the distal end of the tibia/fibula pointing anterior (**Figure 2**). The femoral artery of the donor was attached to the femoral artery of the recipient via an end-end anastomosis. While the vein anastomosis involved and end (donor)-side (recipient) arrangement (**Figure 11**). Importantly, in year 2, we refined the model to produce a less aggressive form of rejection. Initially we had focused on stopping immunosuppression at day 14 and allowing rejection to occur. This resulted in a very fast and strong rejection, which would not be as useful in studies of vasculopathy. After testing various doses of tacrolimus, we found a regimen of full dose (2mg/kg) followed by ¼ dose, (0.5 mg/kg) produces a model with rejection, but with a time course over weeks, not days.

Task 2**: **To establish baseline and experimental imaging standards in rat VCA model. Because the goal is to evaluate vasculopathies in VCA, including the consequences of rejection on vascular integrity, we employed an initial immune-suppression protocol designed to provide baseline information of vascular function during graft healing without rejection, vascular function post-healing without rejection, and vascular function with rejection. In year 2 of the study we are now focusing on a model which gives a full dose of tacrolimus for the first two weeks, followed by maintenance on a ¼ (0.5 mg dose). Assessment of graft health occurs during this entire time course.

In Year 2 we also initiated high-resolution ultrasound imaging of VCA vasculature in the rat model. This required some practice sessions to determine the best probe to use and to set up procedures to transport the animals and safely

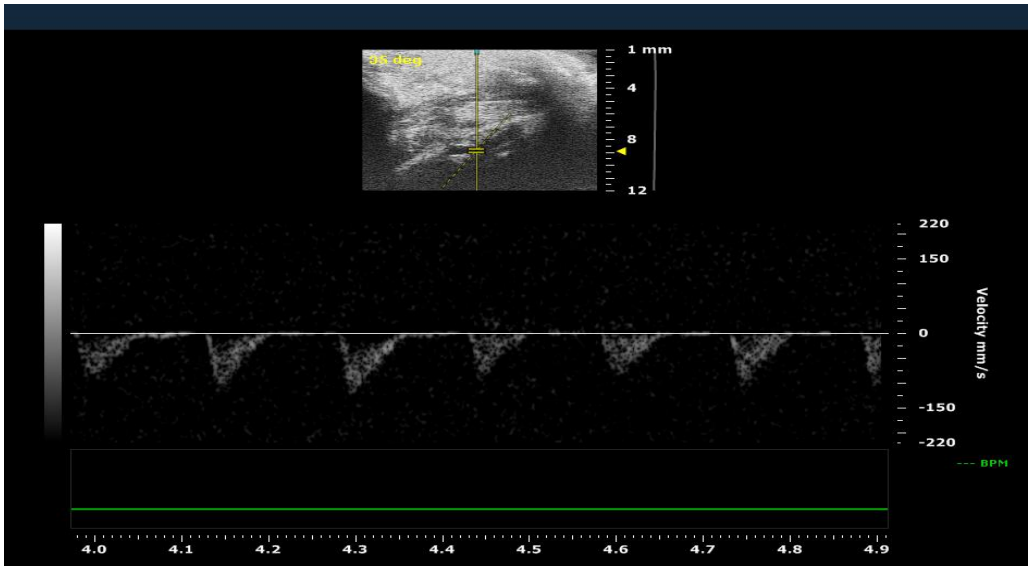


Figure 10

anesthetize them for the imaging procedures. We have now established those and have multiple timepoints at weekly intervals in three rats. While cross sectional images are easier to obtain in the graft, we have been able to obtain longitudinal images as well.

We have also been able to obtain Doppler measurements of blood flow in the main vascular leash to the flap (figure 10) and analyze the leash vessels using the Vevo Vasc software (figure 11).

Additionally we are able to use color Doppler to view blood flow within the flap (figure 12).

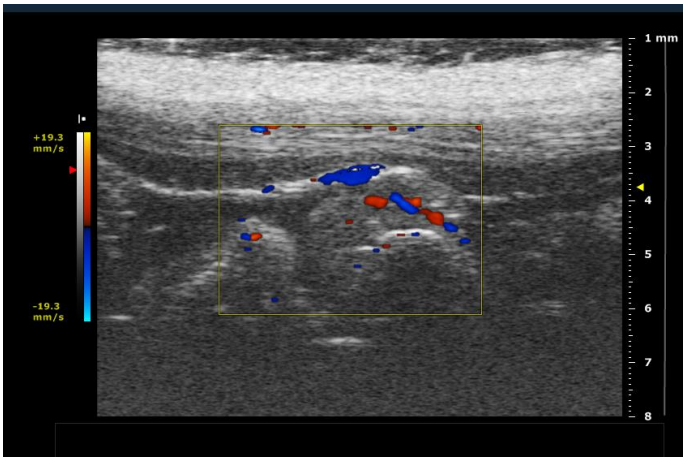


Figure 12: Color Doppler blood flow within OMC flap in Rat #301

Skin-resident T cell isolation—One aspect of VCA immune responses which we wish to examine is the presence and influence donor skin-resident T cells has on the development of rejection and its various complication in VCA. Using the Brown-Norway (RT1.An) -> Lewis (RT1.Al) rat osteomyelocutaneious flap VCA model, we plan to assess T cells within the skin of VCA grafts at varying immune states from active, destructive rejection to quiescent homeostasis. In order to assess the resident skin cells within the donor allograft, we will need to be able to isolate the cells and culture. In addition, we will need to be able to distinguish

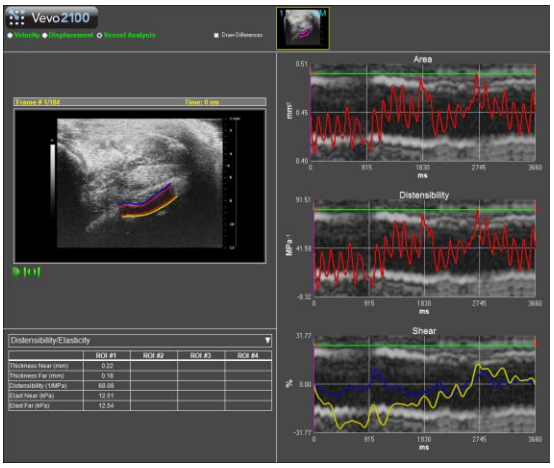


Figure 11 : VevoVasc analysis of the same leash, showing IMT of near and far wall of the vessel, as well as area, distensibility and shear within the vessel.

between donor and recipient derived cells. Following a protocol from Sanchez-Rodriguez et.al. (2014J. Clin. Invest 124:1027-1036), cutaneous fat was removed from BN skin and chopped into the very small pieces before being placed in tissue culture

medium containing collagenase and DNase I digest. The enzymatic digests were incubated at 37C for 18-20h, at which time the tissues were mechanically disrupted by being pushed through a 100 um mesh with a syringe plunger. The digests were then washed in PBS-BSA and further filtered through 70 and 40 um mesh. The resultant populations were counted and stained for T cell and other surface markers. Staining is shown in Figure 13. CD45+ cells from the lymphoid population were gated (left panel) and staining for the rat Class I expression, using biotinylated OX27 mAb (which stains recognizes RT1.Ac and RT1.An expressing cells—middle panel). As shown, once conditions are suitable, ~100% of the CD45+ cells expressed the BN-specific MHC class I molecule. We also stained for CD3+ and MHC class II expression from the CD45+ cells. From the CD45+ cells obtained, ~16% were CD3+, the majority of them co-expressing MHC class II. However, more than half of the cells were MHC class II+/CD3negative. When cell numbers were calculated, and recovery from skin based on surface area, 1.2x10^6 CD3+ cells were recovered from a "VCA-sized" piece of BN skin (2.25 cm^2), or approximately 5.2x10^5 CD3 cells/cm^2.

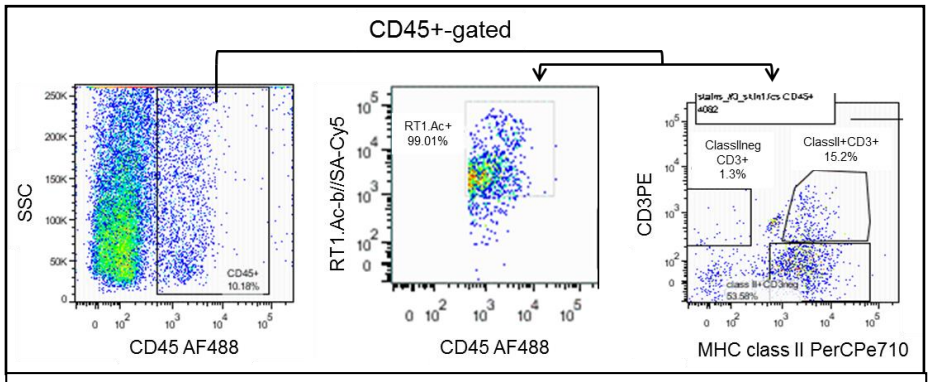


Figure 13. Flow cytometric analysis of BN-digested skin to isolate skin-resident T cells. CD45-gated events (left) were further analyzed for MHC class I expression (RT1A.c(n) middle) or for CD3 and MHC class II (right)

Phenotyping donor and recipient cellular infiltrates in the rat model.

In the osteo-myocutaneous (OMC) flap VCA model using Brown-Norway (RT1.An) donors into Lewis (RT1.Al) recipients, we plan to assess various immune functions, especially T cell function. Because there a donor-specific immune environment is included within these grafts (especially within the bone marrow and the skin) compared to more conventional, single organ grafts, it is imperative that the rat strain of origin is determined when studying the immune activation. To this end, we originally purchased two mAbs from LifeSpan Biosciences which are supposed to differentiate between the RT1.An (clone OX-27; which recognizes RT1.Ac and RT1.An) and RT1.Al (clone OX-3; which had been advertised as a class I mAb for Lewis, Wistar and AO rat strains). When these mAbs were used for FACS analysis of on peripheral blood lymphocytes (PBL) from either BN or Lewis rats, the data were not as expected. Approximately 100% of the BN PBL were positive for RT1.Ac MHC class I mAb and nearly fully negative for the RT1.Bu staining. However, the staining of the Lewis PBL is where the problems are seen. First, although there are positive events for RT1.Bu mAb, only approximately 40% positive. This is not expected of a class I molecule. Upon inquiry, the RT1.B gene is actually analogous to mouse I-A gene, and is therefore, an MHC class II molecule. We have notified the company, and they have since updated their website to correct this product information. The second problem with this data is that the Lewis PBL stains a similar percentage with OX-27 mAb as with the OX-3 mAb. We currently have inquiries to Harlan, who have furnished the rats to us for these experiments, and again to LifeSpan Biosciences. We will also seek another vendor for the OX-27 mAb, because this Ab has been used in many other studies to differentiate between BN and

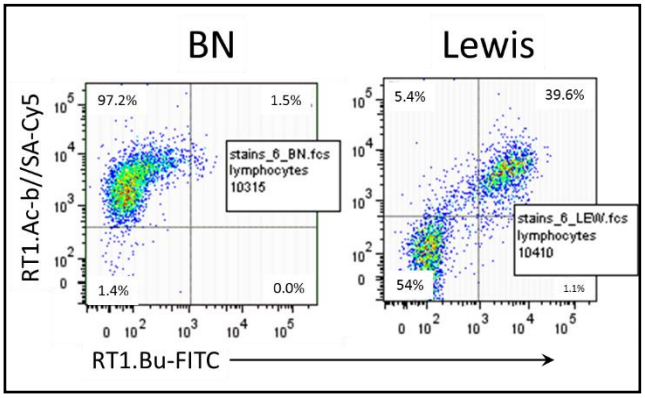


Figure 14: Cross reactivity of class I mAb

Lewis targets, most recently published in a rat VCA model with rats also obtained from Harlan (Plock et al. Adipose- and Bone Marrow-Derived Mesenchymal Stem Cells Prolong Graft Survival in Vascularized Composite Allotransplantation. 2015 *Transplantation* 99:1765-73). We intend to purchase more OX-27 from the same vendor as the Plock et al paper (Serotec). We have not had any luck, so far, finding either a commercially available anti-LEWIS MHC class I Ab source, or a hybridoma from which to grow out mAb. If the search proves to be as futile as it now seems we can one of two things: (1) commission a company to make such a mAb for us, or (2) use a pan-rat MHC class I mAb coupled with the OX-27 to differentiate BN (doubly-stained) from Lewis (singly-stained) cells.

What opportunities for training and professional development has the project provided?

Our hand surgery fellows have had the opportunity to observe our hand transplant recipients, obtain biopsies, participate in surgical planning meetings and practice sessions on cadavers in the fresh tissue lab. Additionally our fellows have gained experience using the Vevo 2100 and LUNA fluorescence angiography systems.

How were the results disseminated to communities of interest?

We have had abstracts accepted to local, national and international meetings including the Tri-state Hand Surgery meeting in Cincinnati, OH, the World Transplant Congress in San Francisco, the American Society of Reconstructive Surgery and the International Hand and Composite Allotransplantation Society meeting which was held in Philadelphia.

What do you plan to do during the next reporting period to accomplish the goals?

In the next quarter we plan on continuing to acqure data. We expect the currently listed hand transplant recipient to receive a transplant before the end of 2015. We have

multiple manuscripts in preparation, including a manuscript on therapy in hand transplant recipients, monitoring of vasculopathy and chronic rejection, and characterization of donor derived T cells in the skin of VCA grafts.

A significant goal in the next quarter is also to obtain and implement timed vibration in our rat OMC flap model. We are currently designing the experiments that we are hypothesizing in conjunction with suboptimal immunosuppression, will induce aggressive vasculopathy in our animal model. We have identified a small animal version of the Theraplate, a vibration device used in the treatment of humans and equines that can deliver low level and high level vibration without requiring us to put the animals under anesthesia.

IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Our initial studies in monitoring subjects using IR-ICG techniques (using the LUNA flourescence angiography unit) suggest that there may be a distinct difference in how the lymphatics drain in transplant recipients vs. normal controls. If we can define these differences, and determine a way to normalize or optimize drainage in transplant recipients, we expect that functional outcomes will be improved. Additionally the ability to monitor nerve function by an anatomic measurement (counting the nerve bundles) may be an important tool to gauge progress in these patients.

The immunomonitoring studies, especially on our patient who is exhibiting signs of chronic rejection in the skin may result in a better understanding of the immune cell populations that are associated with chronic rejection in VCA.

The Vevo 2100 studies will have significant impact on the monitoring of VCA recipients as well as any patients population suffering from vascular disease.

What was the impact on other disciplines?

While the target population of this proposal is VCA recipients, we expect that significantly larger patient populations will also benefit. Cancer patients, especially breast cancer patients will benefit from a better understanding of lymphatic drainage in the upper extremity. In addition, thousands of patients a year have traumatic severing of the digital, medial and ulnar nerves. A technique to monitor recovery would be very helpful in testing interventions to improve nerve recovery in these patients as well.

What was the impact on technology transfer?

These studies have the potential to improve utilization of both fluorescence angiography and high resolution ultrasound through novel applications to understand vessel perfusion, lymphatic drainage and recovery of nerve bundles in ligated and repaired peripheral nerves.

What was the impact on society beyond science and technology?

The goal of this proposal is to improve the quality of life of persons who receive a VCA transplant for the treatment of catastrophic tissue loss. While we are striving to measure, improve and standardize this treatment modality, the ultimate judgment of whether the lives of these patients are restored are made by the patients and their families. If we can play some role in restoring the limbs or face of a soldier to as "normal" as possible, and allow them the best opportunity to integrate back in society, our goal will be achieved.

CHANGES/PROBLEMS:

Changes in approach and reasons for change.

When we initated the monitoring of the rat model, the manufacturer of the Vevo 2100 discouraged us from our plan of routinely rolling the unit from the CMKI 8th floor over to the animal facility one block away. As such we requested permission from the Animal facility to transport the animals from the facility over to the Vevo 2100 unit, in a

laboratory environment on the 8th floor. We set up an area to put the animals under anesthesia for the duration of the imaging procedure. The room and the transport procedures were reviewed and approved by our local IACUC committee.

Actual or anticipated problems or delays and actions or plans to resolve them

The major delay in these studies will be the accrual of VCA recipients. We cannot control the number of patients transplanted. In our clinical trial of hand transplantation (funded outside the current grant) we ar aggressively screening candidates and listing them for transplantation as they are approved. We have a patient who has been listed for hand transplantation for over a year, for whom we have not been able to find a donor because of preformed antibodies. We are hopeful that the recent implementation of UNOS oversight, and the ability to list this patient nationally will increase her chances of finding a donor. With the additional patients we plan to list, we are confident we will have additional VCA recipients to monitor.

Changes that had a significant impact on expenditures

The most significant impact on expenditures have been changes in personnel.

In year 2 of the project we were able to find a post doctoral fellow to replace Dr. Zheng who unfortunately had to be dismissed in August of 2014. We advertised and interviewed several individuals and hired Dr. Steven Mathis, who did not start until February of 2015. Despite excellent recommendations and conversations with his direct supervisor, Dr. Mathis proved to be inadequate prepared to perform the required experiments, and did not demonstrate an aptitude to learn. As such, Dr. Mathis was dismissed after only 3 months. In June of 2015 we were able to hire Dr. Paula Chilton. Dr. Chilton has happily proven herself as an excellent worker and has made significant progress in both the human and experimental studies proposed. Dr. Chilton has provided effort for four months of year 2.

Additionally in Year 2 of the project, Dr. Kutz, one of the co-investigators on the study, became ill, and went on medical leave. We stopped charging his effort to the grant when he was on medical leave, which started in March of 2015. Unfortunately Dr. Kutz's health has not improved and he has stepped down from all duties and has retired. We plan to replace Dr. Kutz's position with Dr. Tuna Ozyurekoglu, who is the current lead reconstructive surgeon for the Louisville VCA Program.

And in a year of changes, our long time nurse coordinator, Brenda Blair, decided to retire, and she was replaced in April of 2015 by Donna Stacy RN.

Significant changes in use or care of human subjects – None to report
Significant changes in use or care of vertebrate animals. – None to report other than the approval to transport the rats to and from the Vevo 2100 for imaging studies.

Significant changes in use of biohazards and/or select agents – N/A

PRODUCTS:

"Nothing to Report."

Publications, conference papers, and presentations

Presentation	Update on Louisville VCA Program - American Society of Reconstructive Transplantation, Chicago, IL, Nov 20-22, 2014
Presentation	Possibilities and Complications in VCA, American Society of Reconstructive Transplantation, Chicago, IL, Nov 20-22, 2014
Presentation	Current Immunotherapy Regimens, American Society of Reconstructive Transplantation, Chicago, IL, Nov 20-22, 2014
Chapter	Kaufman CL, Ouseph R, Kutz JE, Manon-Matos Y, Tien HY, Blair B, Marvin MR, Chapter 13: Chronic Rejection in Reconstructive Transplantation. Brandacher G (ed) The Science of Reconstructive Transplantation. Springer 2015.
Chapter	Huey Y. Tien MD, Yorell Manon-Matos MD, Tsu-Min Tsai MD, Christina L. Kaufman PhD and Joseph E. Kutz MD. Chapter 2: Clinical Pearls and Pitfalls in Reconstructive Transplantation. Brandacher G (ed) The Science

	of Reconstructive Transplantation. Springer 2015.
Presentation	Valyear, K.F., Philip, B.A., Kaufman, C., Kutz, J., & Frey, S.H. Cortical reorganization and recovery of limb function following allogeneic hand transplantation. Extremity War Injury X: Return to Health and Function. January 26-28, 2015. Washington, DC.
Manuscript	CL. Kaufman, MR. Marvin, PM. Chilton, JB. Hoying, SK. Williams, H Tien , T Ozyurekoglu and R Ouseph. Immunobiology of VCA, Transplant International, submitted
Presentation	C.L. Kaufman, R. Ouseph, J.E. Kutz , H.Y. Tien, Y. Manon-Matos, B. Blair, and M.R. Marvin "Incidence of successful steroid weaning in a series of eight hand transplant recipients". Christine M. Kleinert Institute, Transplant Center, Jewish Hospital, University of Louisville and the Kleinert Kutz Hand Care Center, Louisville, KY American Society for Reconstructive Transplantation 4th Biennial Meeting, November 20-22, 2014, at the Drake Hotel in Chicago, Illinois.
Poster	CL Kaufman, R. Ouseph , T. Ozyurekoglu, HY Tien, E. Galvis, Y Manon Matos, M. Palazzo and MR Marvin. Graft rejection in hand transplant recipients with grade 0 skin biopsy histology. 2015 Banff-CST Joint Scientific Meeting, Vancouever, BC
Presentation	CL Kaufman, Update on VCA, 40th Annual Meeting of NATCO August 5-8, 2015, Louisville, KY

Website(s) or other Internet site(s) – None to report

Technologies or techniques – None to report

Inventions, patent applications, and/or licenses – None to report

Other Products – None to report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."

Name: Christina Kaufman PhD

Project Role: Primary Investigator

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: 12 months @ 10% effort

Dr. Kaufman has met on a consistent basis with local grant co-investigators and initiated studies on the LUNA IR/ICG unit as well as the Vevo 2100. She also oversees the day to day administration of the grant and the hand transplant protocol with the Louisville VCA Program.

Name: Jay Hoying PhD

Project Role: Co-Investigator

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: 12 months @ 8% effort

Dr. Hoying has met on a consistent basis with local grant co-investigators and obtained approval for the IACUC protocol through the U of L IRB. Dr. Hoying also initiated discussions with the JH/CHI IT department. Dr. Hoying also works with Rob Reed in performing the rat VCA flaps, and the imaging studies in the experimental model.

Name: Joseph E. Kutz MD

Project Role: Co-Investigator

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: 5 months @ 5% effort

Dr. Kutz has met on a consistent basis with local grant co-investigators to discuss details of the clinical monitoring protocols, for the first five months of Year 2. Dr. Kutz went on medical leave as of March 1, and subsequently retired. We plan to add Dr. Tuna Ozyurekoglu as a new co-investigator.

Name: Michael R. Marvin MD
Project Role: Co-Investigator
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 12 months @ 2% effort
Dr. Marvin has met on a consistent basis with local grant co-investigators to discuss details of the clinical monitoring protocols.

Name: Stuart K. Williams PhD
Project Role: Co-Investigator
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 12 months at 8% effort
Dr. Williams has met on a consistent basis with local grant co-investigators to discuss details of the clinical monitoring protocols as well as the IACUC protocols and the imaging studies in the experimental model.

Name: Brenda Blair RN
Project Role: Research Nurse
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 5 months at 10% effort
Ms. Blair has been working on establishing protocols and methods for IRB and working with Drs. Kaufman, Kutz and Marvin on protocols and patient follow up.

Name: Donna Stacy RN
Project Role: Research Nurse
Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: 7 months at 10% effort

Ms. Stacy has been working on establishing protocols and methods for IRB and working with Drs. Kaufman and Marvin on protocols and patient follow up.

Name: Stephen Mathis PhD

Project Role: Post doctoral fellow

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: 3 months at 100% effort

Dr. Mathis joined the project in February of 2015.

Name: Paula Chilton PhD

Project Role: Research Scientist

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: 4 months at 100% effort

Dr. Chilton joined the project in June of 2015, replacing Dr. Stephen Mathis

Name: Robert Reed

Project Role: Technician

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: 12 months at 30% effort

Mr. Reed has been working on establishing protocols and methods for IACUC project and working with Drs. Hoying and Williams on preparation of protocols. He has established and mastered the microsurgical techniques of the rat VCA model with Dr. Hoying.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Yea, as described above, Dr. Kutz was only on the project for 5 months in year 2.

What other organizations were involved as partners?

Provide the following information for each partnership:
Organization Name: Jewish Hospital Foundation (JHF) and Jewish Hospital (Part of KentuckyOne Health)
Location of Organization: Louisville, KY
Partner's contribution to the project – JHF and JH have provided $1.5 million in funding to cover cost of screening, transplanting and patient follow up for hand transplant recipients that are not covered by insurance.

Organization Name: Kleinert Kutz Hand Care Center
Location of Organization: Louisville, KY
Partner's contribution to the project – The Kleinert Kutz Hand Care Center supplies all of the surgeons which perform the actual hand transplants and help to follow the patients post transplant. In addition, KKHCC staff also participate in the screening of potential hand transplant candidates. The surgeons do not charge for these efforts.

SPECIAL REPORTING REQUIREMENTS – None to report

APPENDICES – None to report